Progress Report

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DEVELOPMENT OF NEW MICROSPECTROPHOTOMETRIC INSTRUMENTATION

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INSTRUMENTATION

All cells are involved in the processes of energy: capture, conversion, transfer, and storage in carrying on their metabolism, processes so necessary for maintenance, growth, and reproduction. The cell develops highly specialized differentiated organelles, e.g., chloroplasts, mitochondria that contain specific pigments and enzymes to carry out these processes. For example, chlorophyll in the chloroplasts for photosynthesis, hemoglobin for oxygen transport in the red blood cell, rhodopsins in the retinal rods and cones of the eye for vision. All of these pigment molecules have certain properties in common.

Special optical and microanalytical methods are necessary to study these processes in the living cell and in the particular organelles. As a result, microspectrophotometry has been under continuous development in order to follow the biosynthesis of these pigments in the living cell by recording the spectral change with time upon excitation.

A completely new instrument, designated as M-5, has been under development for the past two years in our laboratory. The objective was to extend the usefulness of the recording microspectrophotometer by increasing the optical resolution, the sensitivity throughout the wavelength scale from the ultraviolet through the visible and to the infrared, reducing the time constants so that the spectral data can be obtained in seconds, and to miniaturize the instrument.

A schematic of the microspectrophotometer M-5 optical system is illustrated in Fig. 8 and a photograph of the instrument in present operation is shown in Fig. 9.

Two types of optics are being used. One is the use of

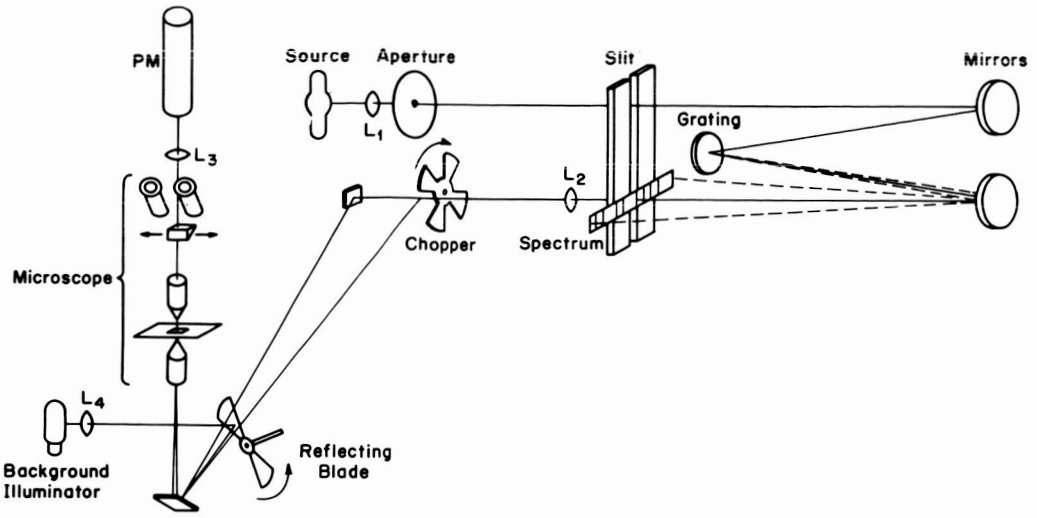

Fig. 8 Microspectrophotometer optical system (M-5).

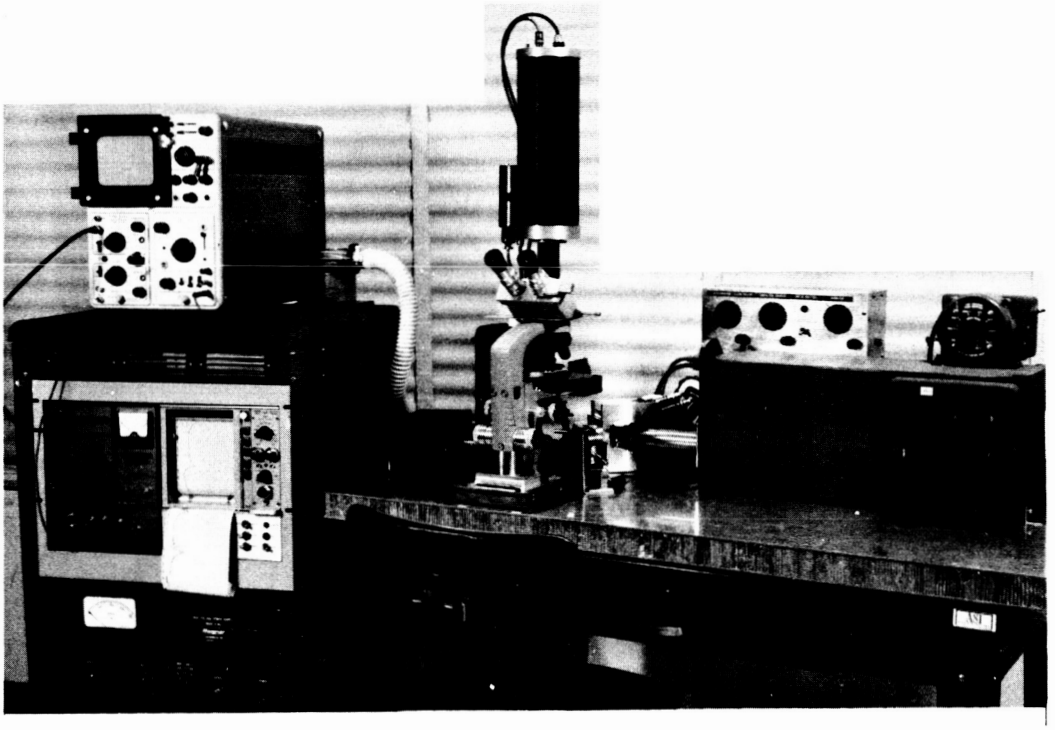

Fig. 9 Microspectrophotometer (M-5).

reflecting optics, which have practically no dependency on wavelength and consequently the focal point does not change with respect to wavelength. They do lack resolving power. The other is the use of Zeiss ultrafluar optics which give better resolving power than the reflecting optics but are limited in their spectral range.

The detector is an EMI 9558Q Photomultiplier with a housing to cool the photomultiplier. The photomultiplier enables the instrument to make measurements with lower levels of light and improves the instrument sensitivity. The EMI 9558Q photomultiplier tube has a spectral response from 162 mμ to 840 mμ, with a quantum efficiency of about 20 per cent from 200 to 400 mμ 10 per cent efficiency at 480 mμ, and 10 per cent efficiency at 785 mμ. The dark current noise is extremely low, and with cooling to -80°C. reduces the dark current about 100 times.

The monochromator used is a Canalco rapid-scanning one, which has 600 line/mm. grating and has ten different speeds, from three seconds to 3,000 seconds (0.2 to 200 mμ per second) over a 200 to 800 mμ range. Its dispersion is 4 mμ/mm. and peaks at 500 mμ. A disadvantage of all grating monochromators is that the light is about 10 to 20 per cent polarized (a part of this polarization can be removed by using a quartz diffusing plate).

The accuracy in measuring the relative percentage of absorption (as far as electronics are concerned) depends on the relative heights of the two pulses; the sample and reference areas are not measured simultaneously but alternately in time.

Therefore, if the overall accuracy is to stay within one per cent, any change in the relative height of the pulses due to relative spectral quantum efficiency of the photocathode, the relative spectral distribution of the light source, or the gain of the amplification should be within one per cent during the time for one pair of pulses. Consequently, a short-scanning time requires a correspondingly short pair-time. The desired short pulse pair-time required the development of a precision, high-speed chopper, Fig. 11 and special electronic circuits in the amplifier, Fig. 10. The chopper in the M-5 has been the main design improvement over previous instruments.

The electronics consist of a preamplifier mounted on the case of the photomultiplier tube, a main amplifier, an electronic switch, an automatic gain amplifier, a feedback circuit, an integrating circuit for each sample and reference pulse, and a panel for necessary controls, Fig. 10.

The lead from the photomultiplier to the preamplifier is as short as possible in order to minimize lead-in capacity. The preamplifier then has a current gain of about -150X. The main amplifier gives the final amplification to the signal, which at this point consists of both sample and references pulses alternately in time. The dc level is restored by a clamping circuit. The electronic switch, synchronized to the chopper, then separates the signal. The reference pulse goes to the automatic gain amplifier, where it is further amplified, and then to the feedback circuit.

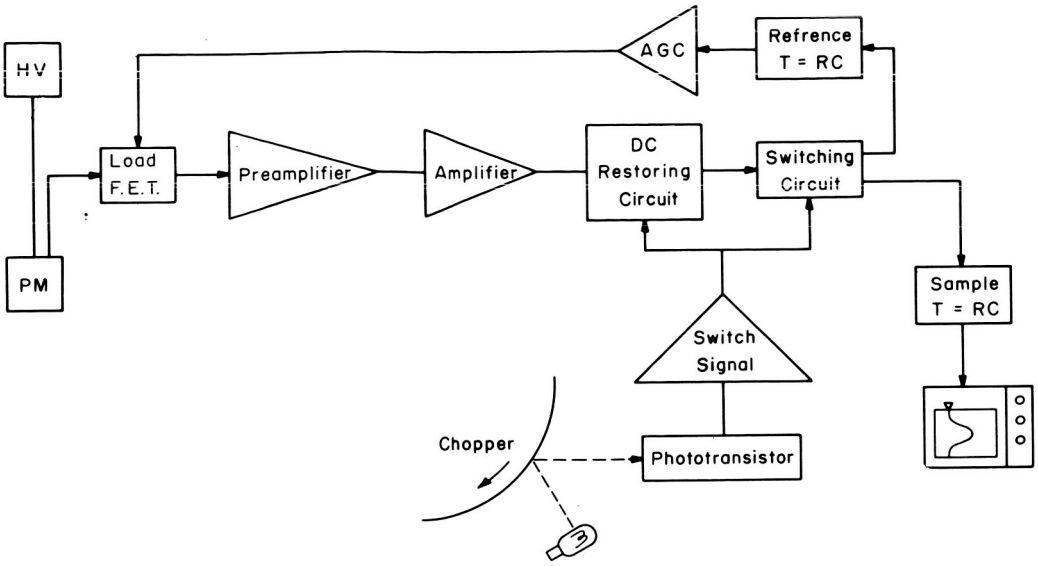

Fig. 10 Microspectrophotometer electronic block diagram.

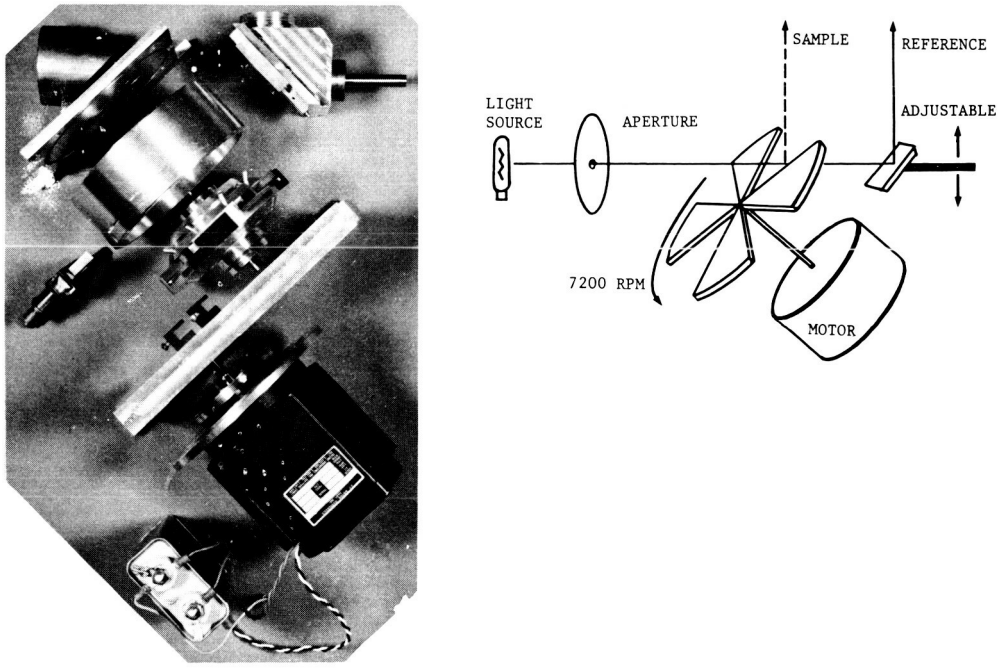

Fig. 11 a, Chopper used in the (M-5) microspectrophotometer.
b, schematic diagram of the chopper.

The feedback circuit consists of a field effect transistor which acts as the load resistance for the photomultiplier. The output from the automatic gain amplifier is connected to the gate of the field effect transistor. The resistance of the field effect transistor depends on the potential of its gate in such a way that low voltage causes a low resistance, and high voltage causes a high resistance. As the signal from the photomultiplier increases, the output of the automatic gain amplifier, which goes to the gate of the field effect transistor, decreases. There is also a corresponding decrease in load resistance which decreases the signal. This operates over a range of 5,000 to 1. The circuit holds the reference pulse constant over a range of 5,000 times within two per cent. The sample pulse is simply integrated and sent to the recorder where the data is plotted.

The recorder for the M-5 is a D'Arsonval-type pen drive, rather than a servomechanism-type recorder used for the M-3 and M-4 instruments. Also the electronics are much more versatile and enhance the response speeds. The new microspectrophotometer, M-5, scans the visible spectrum 400 to 700 mμ in less than 10 seconds or 100 wave numbers in 0.33 seconds. The greatest advantage of this instrument is that it can operate at a low level of light, 10^4 photons per second or a total of 2×10^5 photons for a measurement. The other advantage is that the system is noise limited.

a. Applications of microspectrophotometry.

In our laboratory the M-5 instrument has been applied to studies of: (1) the biosynthesis of pigments, for example, chlorophylls, carotenoids, porphyrins, cytochromes, hemes. All of these pigments are important molecules involved in energy transfer and necessary for life; (2) the spectra in single red blood cells, for the identification of the heme pigments; (3) the spectra and changes upon light excitation in single retinal rods and cones of the eye; and to gain new insight from the spectra about color vision.

Only a few examples taken from our studies are illustrated in Figures 1 e, 3 a, 12, 13, 14, 15, and 16. Particular emphasis was directed towards the identification of pigment molecules, e.g., carotenoids, chlorophylls, porphyrins, hemoglobins. These pigments are directly related to the energetics of living cells; and therefore, the ability to identify them and follow their structural changes by their spectra has much to tell us about these molecules in the life processes.

b. Examples of spectra obtained with the Microspectrophotometer M-5.

(1) One of the applications was to obtain spectral information from single red blood cells, some of the data is tabulated in Table 1 and spectra illustrated in Figures 12, 13, and 14. Note in Fig. 12 an absorption spectrum of a single human red blood cell

Table 1

Absorption Spectra of Single Red Blood Cells

	Major Absorption Peaks		
Human	415	541	577
Rabbit	407	534	572
Mouse	416	544	577
Chick embryo (14 days)	413	543	576
Frog (Rana pipiens)	415	539	572
Turtle (Pseudemys scripta elegans)	408	539	574
Earthworm (plasma)	414	538	567
Water Flea (plasma) (Daphnia pulex)	415	541	574

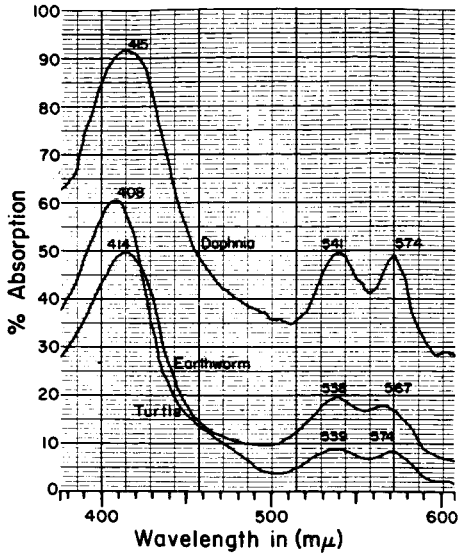

Fig. 13 Daphnia, turtle, and earthworm red blood cell spectra (M-5).

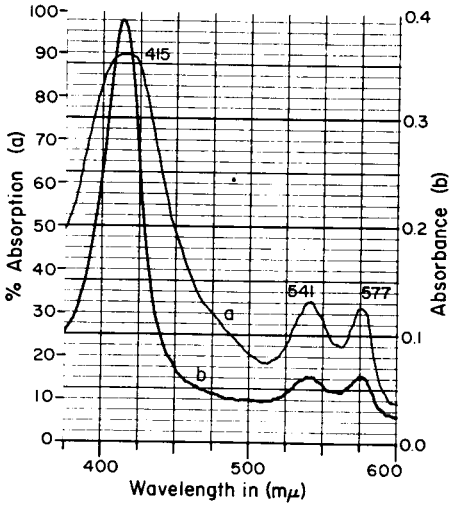

Fig. 12 Human red blood cell spectra. a, micro-spectrophotometer (M-5), single cell. b, Cary spectrophotometer.

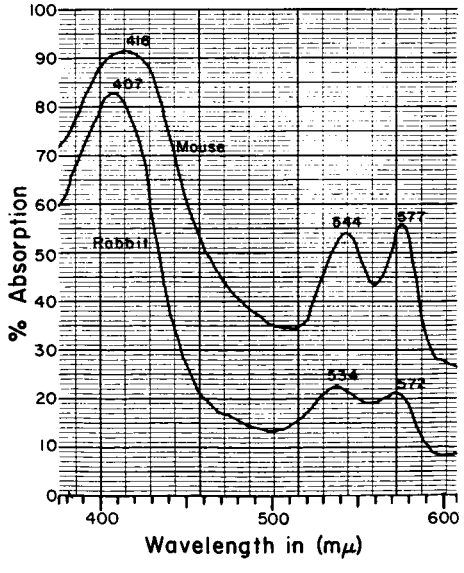

Fig. 14 Mouse and rabbit red blood cell spectra (M-5).

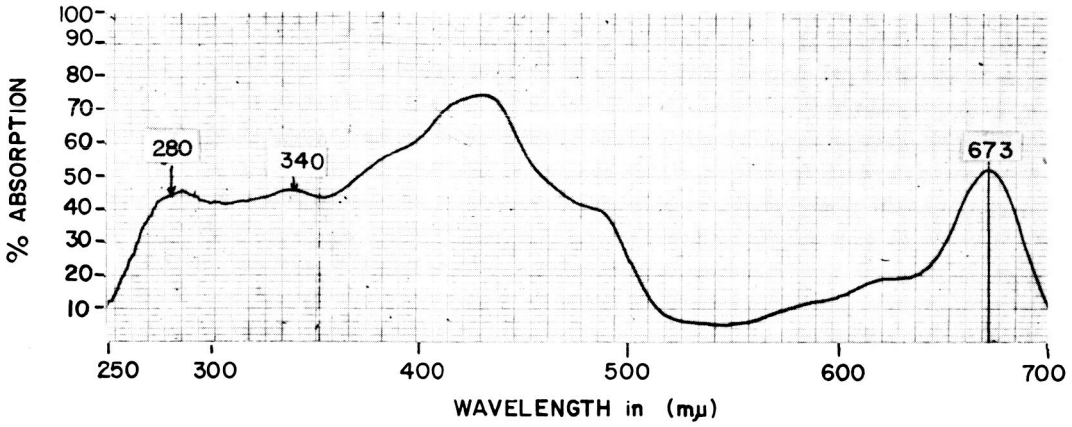

Fig. 15 Light grown Euglena chloroplast spectrum from the microspectrophotometer (M-5).

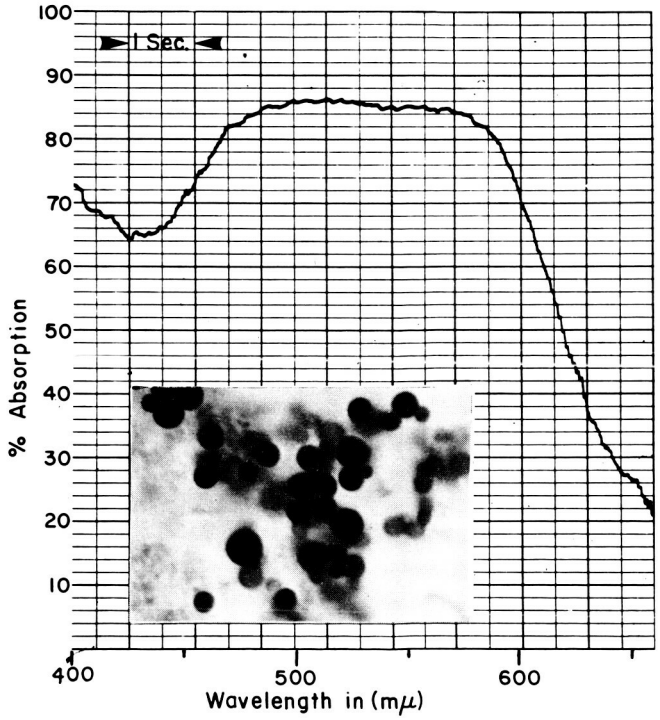

Fig. 16 Spectrum of stained protenoid microspheres (RNA).
a, photomicrograph of microspheres, X 1300.

(a) as compared to a solution of human red blood cells obtained in the Cary-14 spectrophotometer.

(2) A complete spectrum (250 to 750 mμ) of a chloroplast in a living Euglena cell, Fig. 15, showing the ultraviolet peaks and the visible absorption peaks which indicate that most of the chlorophyll in the chloroplast is chlorophyll a.

(3) Proteinoid microspheres with incorporated RNA (prepared according to Dr. S. Fox) stained with methyl green pyronin Y, Fig. 16.

Although the use of microspectrophotometry for the study of the chemistry of living cells, and for the identification of organic molecules is emphasized here, it chould also be applied to the study of oceanic life, oceanic sediments, fossils, meteorites, and extraterrestrial debris.

PUBLICATIONS (September 1, 1966 to May 30, 1967)

Wolken, J.J., "Lipids and the Molecular Structure of Photoreceptors", J. Am. Oil Chem. Soc., 43: 271 (1966).

Wolken, J.J., "Microspectrophotometry", in Encyclopedia of Chemistry, edited by G. L. Clark, Reinhold Publishing Corporation, New York, New York (1966), pp. 452-454.

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RELATED REPORTS TO SCIENTIFIC MEETINGS (September 1, 1966 to May 30, 1967)

September 5-9, 1966. Microspectrophotometry of IN VIVO Pigments and Energy Transfer, Second International Biophysics Vienna, Austria.

October 20 1966. "Third Systems Symposium -- Systems Approach in Biology", sponsored by Case Institute of Technology, Cleveland, Ohio.

October 27 and November 1, 1966. The Physiology of the Eye and Visual Excitation, Biomedical Engineering, University of Pittsburgh School of Engineering, Pittsburgh, Pennsylvania.

December 14 and 15, 1966. Vision: Photoreceptor Systems in Animals, Department of Biophysics and Radiation Biology, University of Rochester, Rochester, New York.

January 10, 1967. Research in Photobiology, Pennsylvania Junior Academy of Science, Antonian Hall, Mount Mercy College, Pittsburgh, Pennsylvania.

February 10 and 13, 1967. Two-part colloquium entitled Photoprocesses of Living Cells, Center for Theoretical Studies, University of Miami, Coral Gables, Florida. Dr. Wolken was Guest Professor at the Center for Theoretical Studies during the month of February 1967.

February 16 and 17, 1967. Comparative Structure of Photoreceptors, part of special Seminar on Photobiology, Washington, D.C., supported jointly by the Consortium of Universities of the Washington Area and the Smithsonian Institution, Washington, D.C.

February 22-25, 1967. Purification of Cattle Rhodopsin by Immunological Purification, 11th Annual Meeting of the Biophysical Society, Houston, Texas. (With G.J. Gallik and R.A. Cornesky).

April 5 and 6, 1967. Photoprocesses in Biology and Research in Biology, University of West Virginia, Biological Sciences Program in Centennial Series, Morgantown, West Virginia.

May 7-10, 1967. Cellular Organelles and Lipids, Annual Meeting of the American Oil Chemists' Society, Symposium entitled "Lipid Monolayer and Bilayer Models and Cellular Membranes", New Orleans, Louisiana.

May 11, 1967. From Thomas Huxley to Molecular Biology, Society of Sigma Xi, Duquesne University, Pittsburgh, Pennsylvania.

May 30 and 31, 1967. The Photoreceptors of Arthropod Eyes, "Symposium on Invertebrate Receptors", Zoological Society of London, London, England.